

HYPSORHODOPSIN: THE FIRST INTERMEDIATE OF THE PHOTOCHEMICAL PROCESS IN VISION

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1. Introduction

The primary processes in visual excitation are initiated by a photochemical event, the absorption of a photon by the photoreceptor, rhodopsin, resulting in successive thermal reaction forming several intermediates. Spectral changes due to the formation of these intermediates after excitation of the rhodopsin occur very rapidly at physiological temperature. The spectral changes have been observed by freezing samples in glass-forming solvents at low temperatures where interconversion rates are so slow that ordinary absorption spectroscopy suffices. By the low temperature method, bathorhodopsin (formerly called prelumi-rhodopsin) [1,2] and hypsorhodopsin [2] were observed by irradiating bovine rhodopsin at 77 K and 4 K, respectively. Since the first observation of hypsorhodopsin, it has been a very important question whether hypsorhodopsin or bathorhodopsin is the first intermediate after the absorption of the photon by the photoreceptor, rhodopsin. The room temperature kinetics of formation and decay of an intermediate species absorbing at 560 nm were investigated [3] using picosecond spectroscopy. Using the second harmonic of a Nd/glass laser (fwhm 6 ps, 530 nm) as a picosecond excitation light source, it was concluded that after picosecond excitation of bovine rhodopsin solubilized in LDAO (Ammonyx LO) detergent, bathorhodopsin was formed within 6 ps [4]. Using a 20 ps step echelon, hypsorhodopsin was not found to be formed by 530 nm excitation of bovine rhodopsin in LDAO [5].

Squid hypsorhodopsin has been observed at room temperature with the use of picosecond ruby laser

[6,7]. Hypsorhodopsin was not observed for cattle rhodopsin [4] while it was observed and found to have 50 ps lifetime at room temperature for squid rhodopsin [6,7].

Now the question arises: why there exists the difference in the primary process of vision between cattle rhodopsin solubilized in LDAO detergent excited by 530 nm pulse and squid rhodopsin solubilized in digitonin detergent excited by 347 nm pulse? In order to solve the problem we tried picosecond laser flash photolysis of cattle rhodopsin using a 530 nm laser pulse from a mode-locked Nd/glass laser.

2. Experimental

Rod outer segments were isolated from frozen bovine retina obtained from G. Hormel Co. and were solubilized in 0.3 M LDAO (Onyx Chem. Co.), 0.01 M *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.0 by the method in [8]. The other samples were solubilized in 0.3 M octylglucoside at pH 7.0.

The samples were kept under complete darkness and at 2–4°C in a refrigerator. Special care was taken to shield the samples from all spurious light emitted by the flash lamps of the oscillator and the amplifier. All kinetic measurements were made with samples in cells with 2 mm optical pathlength.

The spectral changes were observed by a double-beam picosecond spectroscopy apparatus [9]. All samples were excited at 530 nm with a single 6 ps pulse with total energy of ~0.6–1 mJ. We estimate

that <15% of the sample in the optical path were bleached per excitation pulse. For each kinetic observation at a given wavelength, the sample was stirred between successive shots and completely replaced after 8–10 excitation shots.

3. Results

Figures 1–3 show the time dependence of absorbance change at 430 and 440 nm (fig.1), 570 nm (fig.2) and 630 nm (fig.3) for bovine rhodopsin in LDAO and octylglucoside solubilized solutions after a picosecond pulse excitation at 530 nm with 6 ps pulsewidth.

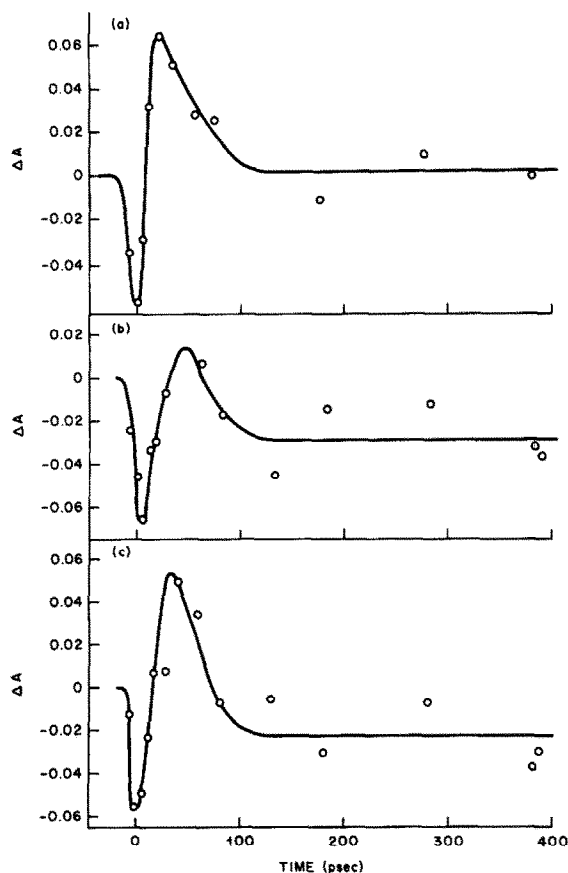


Fig.1. Time dependence of the absorbance change at 430 nm induced by 530 nm picosecond pulse excitation for octylglucoside (a) and LDAO (c) solubilized rhodopsin. Absorbance change at 440 nm for octylglucoside sample is shown by (b).

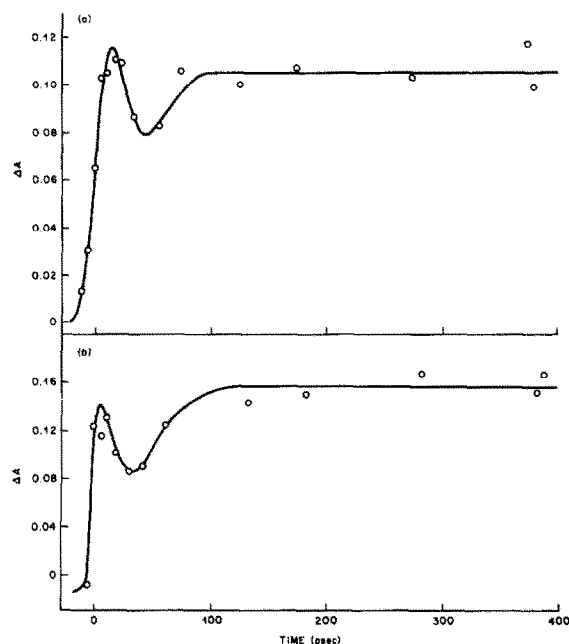


Fig.2. Time dependence of the absorbance change at 570 nm for octylglucoside (a) and LDAO sample (b).

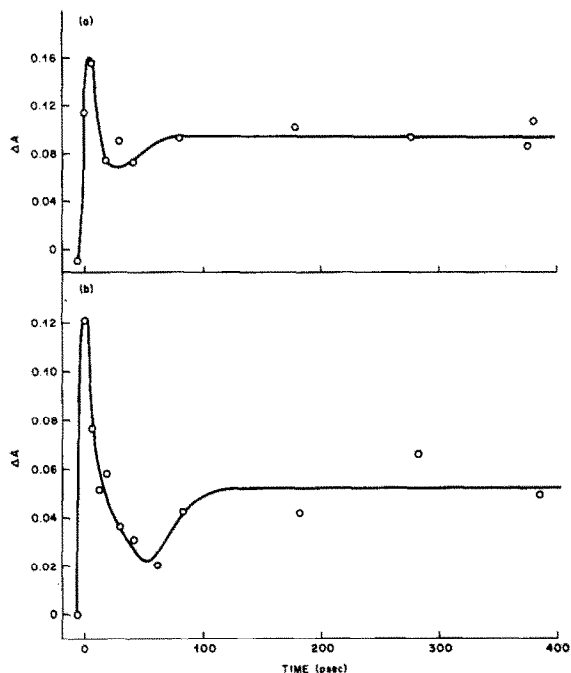


Fig.3. Time dependence of the absorbance change at 600 nm for octylglucoside (a) and LDAO sample (b).

The excitation energy was changed between 0.3 mJ and 1 mJ, and there was no change in the kinetics of absorbance change (ΔA) and ΔA was nearly proportional to the excitation laser pulse energy within experimental error.

The main common feature of the kinetics at 6 different wavelengths (430, 480, 550, 570, 600 and 630 nm) for the LDAO sample and of those at 7 different wavelengths (430, 440, 480, 550, 570, 600 and 630 nm) for octylglucoside sample is that the kinetics is triphasic: there exists the first phase at 0–20 ps, the second phase at 20–80 ps and the last phase at 80–400 ps after excitation.

4. Discussion

As mentioned previously, there are 3 stages in the course of the kinetics of transient species. We tentatively call the intermediates which are dominant at the first, second, and final stages after excitation *X*, *Y* and *Z*, respectively. The time constants for *X* → *Y* and *Y* → *Z* are 15 ± 5 ps and 50 ± 20 ps, respectively.

Figure 4 shows the time-resolved difference spectra between rhodopsin and transient species at around 10 ps and 40 ps after excitation and an average difference spectrum between rhodopsin and transient species at 80–400 ps after excitation. The two difference spectra observed at 10 ps and 80–400 ps after excitation are similar to that between the bathorhodopsin and rhodopsin at low temperatures. Therefore, either *X* or *Z* is expected to be bathorhodopsin. However, since the lifetime of *X* is only 10–20 ps, it cannot be bathorhodopsin which is known to have a lifetime of 30 ns [3]. Therefore *Z* can safely be attributed to bathorhodopsin. *Y* is attributed to hypsorhodopsin because the difference spectrum between hypsorhodopsin and rhodopsin at low temperature resembles that observed at 40 ps after excitation except in the wavelength region 530–630 nm. In this spectral region the absorption spectra of *X* species and bathorhodopsin (*Z* species) affect the spectrum. *X* species is the precursor of hypsorhodopsin and it can be safely attributed to the lowest excited singlet state of rhodopsin.

From the temperature dependence of bathorhodopsin formation and the deuterium isotope substitution effect, proton translocation was claimed to be

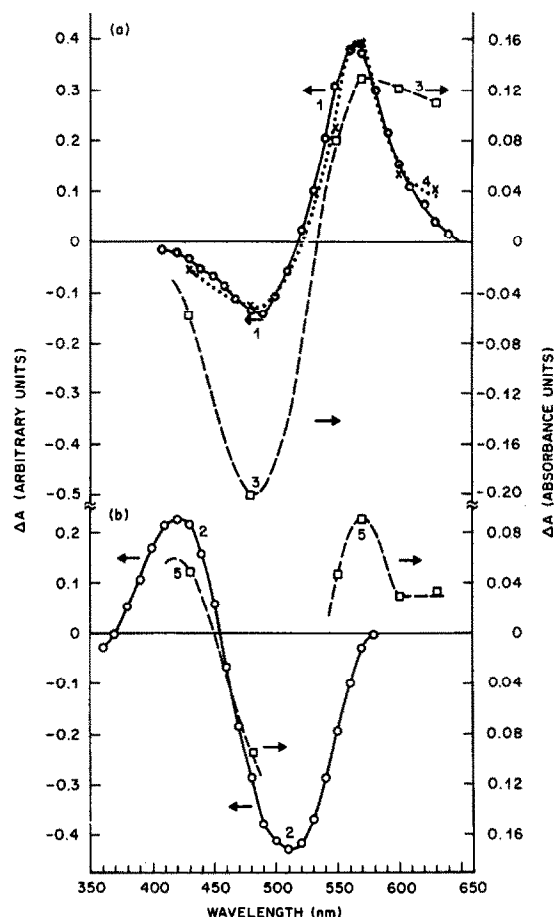


Fig.4. Time-resolved difference spectra between rhodopsin and transient species at ~10 ps (curve 3) and 40 ps (curve 5) after excitation for octylglucoside sample. Difference spectrum between rhodopsin and transient species averaged over 80–450 ps time region after excitation (curve 4). Difference spectrum between rhodopsin and hypsorhodopsin (curve 2) and that between rhodopsin and bathorhodopsin (curve 1) at low temperature for octylglucoside sample.

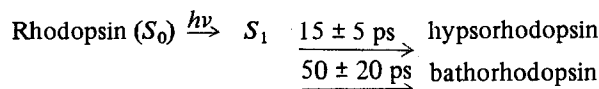
the photochemical process of rhodopsin excited state → bathorhodopsin [5]. With the use of a 20 ps pulse of the second harmonic of ruby laser (347 nm) hypsorhodopsin was observed to be formed from squid rhodopsin in solution with digitonin as a detergent with a time constant <20 ps at physiological temperature and it converts to bathorhodopsin with a time constant of 50 ± 10 ps [6,7]. The discrepancy between the formation time constants of bathorhodopsin for cattle (<6 ps) and for squid (50 ps) was

previously interpreted as possibly due to the difference in the excitation wavelength, in the detergent or in the protein itself. Here however, we found that hypsorhodopsin is the main first intermediate of bovine rhodopsin solubilized in LDAO and octylglucoside excited by 530 nm pulse and the time constant of 50 ± 20 ps was found to be the same as that for squid rhodopsin. The percentage of direct formation from the excited state of rhodopsin was estimated to be $7^{+18}_{-7}\%$ for the octylglucoside samples and $0^{+10}_{-0}\%$ for the LDAO samples.

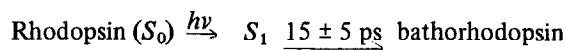
The existence of hypsorhodopsin was not noticed [4] because of the difficulty caused by the resemblance in the absorbance at 570 nm between the excited singlet state of rhodopsin and that of the ground state of bathorhodopsin [4]. As can be seen from fig.2, it is very difficult to recognize the shallow dip in the curve of the time dependence of absorbance change at 570 nm where ΔA was observed [4]. At low temperature (<20 K), they could see the decay of the excited singlet state of rhodopsin. The spectral changes of rhodopsin excited state and bathorhodopsin caused by temperature decrease may indicate that the decay is easier to be seen at low temperature than at room temperature.

Since there are 3 species which are concerned in the kinetics of rhodopsin in the time region of 0–400 ps after excitation, kinetics and time-resolved spectra are complicated. Therefore the low temperature experiment must be repeated since hypsorhodopsin was not detected [4] and it is of utmost importance for the study of the formation mechanism of hypsorhodopsin and bathorhodopsin to see the temporal behavior of three species, i.e., rhodopsin in the excited state, hypsorhodopsin and bathorhodopsin.

As a conclusion it may be stated that the first evidence was obtained that the bathorhodopsin is mainly formed by the following process:



There may be a smaller portion ($<25\%$) of bathorhodopsin formed by the following process:



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